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RESEARCH ARTICLE

Endophytic bacteria from paddy with double 1-aminocyclopropane-1-carboxylic acid deaminase and nitrogenase activity

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ABSTRACT

Plant growth promoting bacteria with dual activity, 1-aminocyclopropane-1-carboxylic-acid deaminase (ACCD) and nitrogenase, is more effective in supporting plant growth under stress condition. Previously, we were obtained several endophytic bacterial strains that exhibited dual activity, one of which was *Raoultella terrigena* PCM8. This study aimed to characterize the ACCD and nitrogenase genes of PCM8 strain. The *acd*S gene was obtained from the results of Whole Genomic Sequencing analyis, while the *nif*H gene was obtained by PCR. The characterization of both of the genes was carried out by means of *in-silico* analysis. WGS annotation analysis, showed that the *acd*S gene of PCM8 was located at the locus 19090 of genomic DNA and contains 978 nucleotides. *In silico* analysis of both *acd*S and *nif*H gene products showed that the ACCD enzyme of PCM8 had 325 amino acids, with molecular weight of 34.95 kDa, while nitrogenase as represented by *nif*H subunit product consist of 96 amino acids with molecular weight of 93.98 kDa, respectively. The ACCD had pI value of 5.06, and catalytic residues of Lys51, Ser78, Tyr287, and Thr288, while *nif*H gene product had the pI value of 11.77. The results suggested that *R. terrigena* PCM8 potentially produce double activity of ACCD and nitrogenase and therefore it can be a good candidate as plant growth promoting under stress condition.

Keywords: *acd*S gene; 1-aminocyclopropane-1-carboxylic acid deaminase; endophytic bacteria; *nif*H gene; nitrogenase *Raoultella terrigena*

INTRODUCTION

Ethylene is the one of phytohormone that required to promote plant growth. However, at high concentration ethylene has detrimental effect on the plant growth (Iqbal et al., 2017). The stress conditions such as extreme temperature, flooding, drought, heavy metals, high salinity and pathogenic attack, can induce high production of ethylene. The increasing of ethylene concentrations is caused by production of, ethylene biosynthetic precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), as a response to the environmental stress.

One way to control ethylene concentration is to convert the ethylene biosynthetic precursor, ACC, into ammonia and α-ketobutyrate which can be assisted by microbes producing ACCD enzymes. The enzymatic activity of ACCD, a PLP-dependent enzyme, can be determined by measuring the amount of ammonia and alpha ketobutyrate formed after incubation on ACC substrate as the sole nitrogen source. In our previous study, we found 11 endophytic bacterial isolates that have the potential to produce ACCD enzymes, one of which is PCM8 isolate. The 16S rRNA gene analysis showed that PCM 8 was closely related to *Raoultella terrigena*. The ACCD-harboring rhizobacteria and the presence of the corresponding gene have been studied extensively at the biochemical and molecular level. However, there only a few reports on study of endophytic bacteria harboring ACCD gene.

In our earlier study, ammonia concentration measurements conducted to screening ACCD activity of endophytic bacteria

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isolates. Measurement of α- ketobutyrate concentration formed were conducted to confirm the ACCD activity from the previous study. Confirming the bacteria producing ACCD can also be done through detection of the ACCD enzyme coding gene, the *acd*S gene. Amplification of the *acd*S gene by PCR with degenerate primers has been widely used for molecular identification of ACCD-producing bacteria (Govindasamy et al. 2008; Onofre-Lemus et al., 2009; Nikolic et al., 2011). The *acd*S gene is under the control of a regulatory gene (*acd*R), which is transcribed in the opposite direction of the *acd*S gene and encodes an Lrp (leucine-responsive regulatory protein) that is necessary for *acd*S expression (Li and Glick, 2001). Nucleotide sequences of the *acd*S gene are very close to other genes namely *dcy*D and *yed*O which encode for another PLP-dependent enzyme D-cysteine sulfhydralase (Singh et al., 2015).

Since the low conservation of gene sequence among the *acd*S genes, it is very hard to isolate the *acd*S gene from bacterial genome. Therefore, screening of the *acd*S gene with whole genome sequencing (WGS) is necessary to be done to determine the sequence of the *acd*S gene in PCM8 isolates. Gene of *acd*S can be found in both Gram-negative and Gram-positive bacteria (Belimov et al., 2001), (Ma et al., 2003b), (Rashid et al., 2012), and fungi (Jia et al., 1999). *acd*S gene generally has approximately 900-1200 bp in length.

Similarly, nitrogen also plays significant roles in the plant growth and metabolism (Raymond et al., 2004). The nitrogen can be supplied from nitrogen sources in the form of organic compounds and N_2 (dinitrogenase) gas. Various types of bacteria could fix N_2 gas to produce the organic nitrogen that could support plant in term of nitrogen requirement. The enzymes that responsible for nitrogen fixation are nitrogenases. This enzyme is conservative because it has the same gene structure, but the expression is different.

Nitrogenase is an enzyme which has ability to breakdown the triple bond of nitrogen gas into ammonia. Nitrogenase is divided into two main groups, namely dinitrogen reductase which has a Fe protein molecule and dinitrogenase which has a molecule of Mo-Fe protein (Dighe et al., 2010). In addition, nitrogenase also catalyze the reduction of acetylene (C_2H_2) to ethylene (C_2H_4). Nitrogenase activity can be measured by the Acetylene Reduction Assay (ARA) method by measuring reduced acetylene (Hardy et al., 1968).

Nitrogen fixing bacteria can be identified based on the presence of *nif* gene in their genome (Franche, 2009 and Reinhardt et al., 2008). The *nif* genes that are important for nitrogenase synthesis have a 24 kb cluster (Li and Chen, 2020). The *nif*H encodes the nitrogenase protein subunit, which is the most conserved part of the whole group of nitrogen fixing genes and it is used as an ideal molecular marker for microorganisms (Deslippe and Egger, 2006). The *nif*H gene consists of parts that are conserved and can be amplified using primary degenerate (Gaby and Buckley, 2012).

The *nif*H gene product functions as a component of the nitrogenase system and has a role in the formation of Fe-protein complex. Therefore, it can be concluded that the presence of the *nif*H gene indicates the existence of a nitrogenase system and the ability to fixing molecular nitrogen (Franche et al., 2009).

Some ACCD-producing bacteria are also known to have another activity. Bacterial strain that has ability to produce ACCD and nitrogenase simultaneously will increase plant growth more effectively compared to the sole activity of ACCD or nitrogenase. Some of the bacterial strains that have been disclosed are WAN1 in wheat crops (Hassan et al., 2015) and SAN1 strains under Cadmium metal stress (Hassan et al., 2016). This study aimed to isolate, identify and characterize of *acd*S and *nif*H gene from *R. terrigena* PCM 8.

MATERIALS AND METHODS

Bacterial strains

One plant growth-promoting endophytic bacteria (PGPEB) that exhibited ACCD and nitrogenase activity, *Raoultella terrigena* PCM 8 (OR405948), that was previously shown to ameliorate of salt tolerance in soybean (*Glycine max* L.) was used in this study (Simarmata et al, 2018). The bacterial strain was cultured and enriched in Tryptic Soybean Agar (TSA) and Tryptic Soybean Broth (TSB).

Morphological identification

Morphological identification conducted by examining bacterial colonies and cells. Bacterial isolates were grown on NA medium, then the growing colonies were examined base on color, shape, and edge. The cell morphology was examined under microscopic analysis. Gram staining was carried out to determine whether the bacterial isolate is either Gram-positive or Gram-negative bacteria (Hiremath and Bannigidad, 2011).

ACCD enzyme activity

Selection of ACCD-producing bacteria was performed by measuring ACCD activity and isolating the *acd*S gene. Bacterial were grown in Dworkin-Foster's (DF) salt minimal medium supplemented with aminoisobutyric acid (AIB). Subsequently, bacterial cells and supernatant were separated by centrifugation (Eppendorf 5430 R. Germany).

The collected ACCD cytoplasmic proteins containing supernatants were used for ACCD enzyme activity assay (Penrose et al., 2003).

Measurement of cytoplasmic protein concentration was carried out by Biorrad Assay method. A total of 2 µL crude enzymes was mixed with 200 µL Biorrad solution. The mixture was mess up to 1000 µl with aquadest and homogenized. The mixture was then measured at absorbance of 595 nm. BSA (*Bovine Serum Albumin*) was used as standard protein (Bradford, 1976).

The ACCD activity of the bacteria performed as follows: 200 µL of cytoplasmic protein and 20 µL 0.5 M of ACC substrate were homogenized and incubated at 30°C for 15 min. Thereafter, 1 mL 0,56 M HCl was added and then centrifuged at 16000 × g for 5 min. 1 mL supernatant was added with 800 µL 0,56 M HCl and 300 µL dinitrophenyl hydrizyl (0,2% in 2 M HCl). The samples were homogenized with vortex Thermo Scientific 88882010 and incubated at 30ºC for 30 min and then added 2 mL 2N NaOH. The color change that is formed shows the reaction of dinitrophenyl hydrazine with α-ketobutyrate formed. Colorimetric analysis was performed with a spectrophotometer (UV-1660PC. VWR. China) at 540 nm wavelength.

Nitrogenase enzyme activity

Measuring of nitrogenase activity was carried out by ARA (*Acetylene Reduction Assay*) method. The bacterial strains were grown in DF salt medium for 24 hours (Dworkin and Foster, 1958). The bacterial cells were harvested by centrifugation at 16000 × g for 5 min. Bacterial pellets were transferred to the venoject tube for nitrogenase activity and others were used for measuring the dry weight of the cell. The cell pellet containing venoject was filled with 10% volume of acetylene (PT. Samator Gas Industri, Tangerang, Indonesia.). After 24 hours the air in the venoject tube was injected into the GC Shimatzu GC-8. The columns used was alumina (1 m × 3 mm) with temperature conditions of column 100°C, injector temperature 200-220°C, nitrogen as carrier gas (N_2), pressure ½ kg/cm^2 and FID (*Flame Ionization Detector*) detector.

DNA Genome isolation

Genome isolation was carried out using the Presto™ Mini gDNA Bacteria Kit isolation kit according to the protocol. The DNA genome was then stored in -20°C.

Whole genome sequencing (WGS)

The DNA genome was also used for Whole Genome Sequencing analysis. WGS is performed using the services of PT. Genetika Science Indonesia with the GridION nanopore sequencing platform from Oxford Nanopore Technologies (Nicholls et al., 2019).

ANI value analysis and WGS annotation

The ANI (Average Nucleotide Identity) values of the sequencing results were then analyzed via jspecies.ribohost.com to determine the genetic relationship. The genome functional annotations were analyzed using RAST 2.0.

In silico analysis

The *in-silico* analysis carried out in this study included phylogenetic tree analysis, physicochemical analysis, and protein structure analysis of the *acd*S gene. Amino acid sequence comparisons were performed using ClustalW multiple sequence alignment. Phylogenetic tree reconstruction was constructed by using MEGA 7 software with the neighbor-joining method and evaluated by bootstrap analysis of 1000 times. Physicochemical analysis of proteins was carried out using the ExPaSy ProtParam program (http://web.expasy.org/protparam). Protein modeling and visualization were carried out using SWISS-MODEL (swissmodel.expasy.org) and PyMol 2.4.1.

Detection of *nif*H genes

Bacterial genome was isolated using the Wizard Genom Purification Kit in accordance with the protocol (www.promega.com). Isolation of *nif*H gene was carried out by PCR method using a pair of F*Nif* (5'-GAAGGCBGACTCCACCC-3') and R*Nif* (5'- TGGCGGATBGGCATGGC-3') primers. The FNif and RNif primers are designed based on alignment of several *nif*H gene nucleotide sequences. Twenty-five microliters of PCR mixture consisted of 1 µl of genomic DNA, 12.5 µl mytaq HS Red mixPCR, 2 µl of each primer, and 7.5 µl ddH_2O was used for PCR reaction. The PCR conditions were as follows, initial conditions denaturation of 95°C for 2 minutes, followed by 30–35 cycles consisting of denaturation of 95°C for 30 seconds, annealing 56°C for 30 seconds and elongation of 72°C for 1 minutes and followed by final extension 72°C for 5 minutes. The PCR products were then electrophoresis on 1% fluorosafe containing agarose gel. The band was visualized by UV transilluminator. DNA sequencing was performed by 1st base sequencing services (Malaysia). The sequencing results were analyzed by BLASTX in NCBI genbank.

Phylogenetic analysis of the *acd*S PCM8 gene with other isolates

The *acd*S PCM8 gene was analyzed phylogenetically to evaluate the closeness relationship with other *acd*S genes in the NCBI genbank. Phylogenetic analysis was carried out using MEGAX software.

RESULTS AND DISCUSSION

Morphological identification of PCM8 strains

Morphological identification was carried out to support the results of molecular identification. Based on microscopic

analysis, it was known that the edge of the PCM8 strain was lobate, the colony was circular and the cells were rod-shaped. Gram stain showed that PCM8 strain was Gram-negative. These results indicate that the strains used are in accordance with the characteristics of the bacteria used in the previous study (Simarmata et al. 2019). Morphological identification is carried out as a reference basis to confirm the results of molecular identification. Even so, the classification with this method is weak considering the simple prokaryotic structure, so it is necessary to do another confirmation test.

Cytoplasmic protein extraction

ACCD is an inducible enzyme. Aminoisobutyric acid (AIB) is an ACC analogue which has ability to induce ACCD synthesis (Honma, 1982). Total protein concentration obtained from the PCM8 strain and *Pseudomonas* UW4, with and without AIB induction, is presented in Table 1.

According to Table 1, total protein concentration increases in all strains when induced by AIB. Based on *Paired T-Test* analysis, conducted to observe the significance role of AIB in total protein concentration, showed that PCM8 and UW4 strains experienced significant cytoplasmic protein concentrations after AIB induced. Increased protein concentrations occur presumably because AIB, an ACC analogue compound, may induce ACCD synthesis. This is in accordance with the reported Honma (1982) that AIB 1% can induce 44.5 × 10^{-3} ACCD/mg protein units.

The increasing of cytoplasmic protein concentrations in *Raoultella terrigena* PCM8 strains was a 4-fold increase when induced by AIB. Furthermore, the test used for measuring ACCD formed by AIB-induction is performed by measurement of α-ketobutyrate concentration (Glick, 1995).

ACC deaminase activity

ACCD activity is measured by the number of moles of α-ketobutyrate formed per milligram of protein per hour. The ACCD activity of the PCM 8 strains and *Pseudomonas putida* UW4 is shown in Table 1.

Based on Table 1 and supported by Duncan's analysis shows that there are significant differences in ACCD activity. The enzyme activity of PCM8 strain was 501,13 nmol α-KBT/mg protein/hour then followed by *Pseudomonas* sp. UW4 of 475,43 nmol α-KBT/mg protein/hour. Differences of enzyme activity in different bacteria can be caused by the differences in the number of ACCD enzyme units present per milligram of cytoplasmic protein or the difference in catalytic side activity of the enzymes produced by each bacterial (Glick, 2014).

Table 1: The ACCD activity and the concentration of cytoplasmic proteins with and without AIB Induction

No.	Strain	Concentration of Cytoplasmic Proteins (ug/uL)		ACCD Activity (nmol α-KBT/mg protein/hour)
		Without AIB Induction	With AIB Induction	
1	PCM8	1,245	4,433	503,13
2	UW4	0,887	1,146	475,43

The ACCD activity of the PCM8 and UW4 strains was correlated with increased cytoplasmic protein concentrations in both strains. This data supported the reason that increased of cytoplasmic protein concentrations are due to the presence of ACCD enzyme that synthesized by AIB induction. On the other hand, PCM8 had a high increase in cytoplasm protein concentration after AIB induction, reaching 4-fold, but ACCD activity produced was not correlated with the increasing of the cytoplasmic protein concentration. This is thought to be caused by AIB was able to induce proteins besides that ACCD, such as AIB decomposing enzyme. This enzyme can hydrolyze AIB into several products, one of which is NH_3 (Honma and Shimomura, 1974). NH_3 formed can be used by bacteria as its nitrogen source for growing. The results are increasing the mass of cell cytoplasm proteins that are formed and also detected by BSA.

Identify *acd*S gene of PCM8 isolate

To confirm the ACCD activity, screening of the *acd*S gene in the PCM8 genome was carried out. Based on the results of the sequencing and analysis using the Geneious Prime software, the gene sequences suspected to be *acd*S were obtained. Based on the BLAST protein analysis at NCBI, it was found that the amino acid sequence of the suspected *acd*S isolate PCM8 was similar to that of the d-cys protein (D-cysteine desulfhydrase) *Raoultella terrigena* of 99% (Fig. 1).

Todorovic and Glick (2008) proved that many residues of the d-cys and *acd*S active sites were conserved. According to Soutourina et al. (2001) the amino acid sequence of d- cysteine desulfhydrase is homologous to those of several 1-aminocyclopropane-carboxylate deaminases even though the *E. coli* desulfhydrase does not use 1- aminocyclopropane-1-carboxylate as substrate. The protein D-cysteine desulfhydrase Enterobacteriaceae (WP_048972058.1) was 100% similar to the amino acid sequence ACCD *Enterobacter cloacae* (AOR81964.1) (Fig. 2).

According to Todorovic and Glick (2008) showed that active site of the tomato D-cysteine desulfhydrase conserved with many of the important residues for ACCD activity. These include the lysine residue that binds the co-enzyme and the active site tyrosine residue that stacks with the pyridine ring.

D-cysteine desulfhydrase [Raoultella terrigena]

Sequence ID: WP_115192366.1 **Length:** 328 **Number of Matches:** 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 328 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
659 bits(1700)	0.0	Compositional matrix adjust.	326/328(99%)	327/328(99%)	0/328(0%)

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Query  1    MSLQNLTRFPRLELIGAPTPLEYLPRLSDHLGREIFIKRDDVTPLAMGGNKLRKLEFLAA  60
            MSLQNLTRFPRLELIGAPTPLEYLPRLSDHLGREIFIKRDDVTPLAMGGNKLRKLEFLAA
Sbjct  1    MSLQNLTRFPRLELIGAPTPLEYLPRLSDHLGREIFIKRDDVTPLAMGGNKLRKLEFLAA  60

Query  61   DALREGADTLITAGAIQSNHVRQTAAVAAKLSLHCVALLENPIGTRAENYLTNGNRLLLD  120
            DALREGADTLITAGAIQSNHVRQTAAVAAKL LHCVALLENPIGTRAENYLTNGNRLLLD
Sbjct  61   DALREGADTLITAGAIQSNHVRQTAAVAAKLGLHCVALLENPIGTRAENYLTNGNRLLLD  120

Query  121  LFNTQVEMCDALTDPNAQLEELATRIEAQGYRPYVIPVGGSSALGALGYVESALEIAQQC  180
            LFNTQVEMCDALTDPNAQLEELATRIEAQGYRPYVIPVGGSSALGALGYVESALEIAQQC
Sbjct  121  LFNTQVEMCDALTDPNAQLEELATRIEAQGYRPYVIPVGGSSALGALGYVESALEIAQQC  180

Query  181  EGAVELSSVVVASGSAGTHAGLAVGLEQLMPGAELIGVTVSRKVADQLPKVVALQQAVAN  240
            +GAVELSSVVVASGSAGTHAGLAVGLEQLMPGAELIGVTVSRKVADQLPKVVALQQAVAN
Sbjct  181  KGAVELSSVVVASGSAGTHAGLAVGLEQLMPGAELIGVTVSRKVADQLPKVVALQQAVAN  240

Query  241  SLELQAKAGITLWDDYFAPGYGTPNDEGMEAVKLLAQLEGILLDPVYTGKAMAGLIDGIQ  300
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Sbjct  241  SLELQAKAGITLWDDYFAPGYGTPNDEGMEAVKLLAQLEGILLDPVYTGKAMAGLIDGIQ  300

Query  301  QKRFKDEGPILFIHTGGAPALFAYHPHI  328
            QKRFKDEGPILFIHTGGAPALFAYHPHI
Sbjct  301  QKRFKDEGPILFIHTGGAPALFAYHPHI  328
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Fig 1. Alignment of the predicted amino acid sequence *acd*S PCM8 with the d-cys sequence of *Roultella terrigena* both proteins showed the high homology with 99% identity.

MULTISPECIES: D-cysteine desulfhydrase [Enterobacteriaceae]

Sequence ID: WP_048972058.1 **Length:** 328 **Number of Matches:** 1

See 5 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 328 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
664 bits(1712)	0.0	Compositional matrix adjust.	328/328(100%)	328/328(100%)	0/328(0%)

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Query  1    MSLQNLTRFPRLEFIGAPTPLEYLPRFSDYLGRDIFIKRDDVTPMAMGGNKLRKLEFLAA  60
            MSLQNLTRFPRLEFIGAPTPLEYLPRFSDYLGRDIFIKRDDVTPMAMGGNKLRKLEFLAA
Sbjct  1    MSLQNLTRFPRLEFIGAPTPLEYLPRFSDYLGRDIFIKRDDVTPMAMGGNKLRKLEFLAA  60

Query  61   DALREGADTLVTAGAIQSNHVRQTAAVAAKLGLHCVALLENPIGTRAENYLTNGNRLLLD  120
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Sbjct  61   DALREGADTLVTAGAIQSNHVRQTAAVAAKLGLHCVALLENPIGTRAENYLTNGNRLLLD  120

Query  121  LFNVQVEMVDALTDPTAQLDELATRLEAQGFRPYVIPVGGSNAMGALGYVESALEIAQQC  180
            LFNVQVEMVDALTDPTAQLDELATRLEAQGFRPYVIPVGGSNAMGALGYVESALEIAQQC
Sbjct  121  LFNVQVEMVDALTDPTAQLDELATRLEAQGFRPYVIPVGGSNAMGALGYVESALEIAQQC  180

Query  181  EGAVSLSSVVVASGSAGTHAGLAVGLEHLLPDVELIGVTVSRSVADQKPKVLSLQQAVAE  240
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Sbjct  181  EGAVSLSSVVVASGSAGTHAGLAVGLEHLLPDVELIGVTVSRSVADQKPKVLSLQQAVAE  240

Query  241  QLELKAKADILLWDDYFAPGYGTPNEEGMEAVKLLARLEGILLDPVYTGKAMAGLIDGIA  300
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Sbjct  241  QLELKAKADILLWDDYFAPGYGTPNEEGMEAVKLLARLEGILLDPVYTGKAMAGLIDGIA  300

Query  301  QKRFKDEGPILFVHTGGAPALFAYHPHV  328
            QKRFKDEGPILFVHTGGAPALFAYHPHV
Sbjct  301  QKRFKDEGPILFVHTGGAPALFAYHPHV  328
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Fig 2. Amino acid sequence alignment Enterobacteriaceae protein, D-cysteine desulfhydrase (WP_048972058.1). The protein showed 100% identity to the *Enterobacter cloacae* ACCD amino acid sequence (AOR81964.1).

ANI value analysis results and WGS annotations of the *acd*S gen

After knowing that the PCM8 strain used was the same as the strain from the previous study, whole genome sequencing was carried out to determine whether the strain had the ACCD enzyme coding gene. ANI (Average Nucleotide Identity) value analysis was carried out to determine the identical kinship value of a strain. Goris et al., (2007) compared whole genome sequences with their hybridization values. Paired comparisons of intact genome sequences showed that the ANI of all conserved genes between the two genomes correlated well with 16S rRNA sequence identity and DDH similarity values. The analysis showed that the ANI value of PCM8 was 98,88%

compared to *R. terrigena* strain NBRC 14941 and 98,83% compared to *R. terrigena* strain NCTC 13038 (Table 2). Arahal (2014) states that the ANI value of 95-96% can be equated with a 70% DDH value so that it describes the same species.

RAST analysis aims to obtain information on the function and annotation of a gene. Based on the results of the RAST analysis, it was found that the PCM8 strain had a nucleotide base measuring 5.693.312 bp with a GC content of 57,4%. The results also showed that the PCM8 strain had 393 subsystems with a coding sequence of 5513 (0,096%) and 117 RNA. This result is close to the results of the research of Fazal et al (2019) which states that the *R. terrigena* strain NCTC 13097 has a nucleotide base of 5.574.669 bp and a GC content of 57,3% with 5,386 coding sequences and 119 RNA genes. Even so, this study has not analyzed the genomic function of *R. terrigena* strain NCTC 13097 so it is not known whether this strain has the *acd*S gene.

The *acd*S gene in the PCM8 strain can be determined by looking for the annotation results of its function and location from the WGS analysis data as shown in Table 3.

The annotation results show that the *acd*S gene is in the same locus as the *dcyD* gene that encodes the D-cystein desulfhydrase enzyme. This enzyme catalyzes the chemical reaction of D-cysteine to sulfide, ammonia, and pyruvate. This is in accordance with Singh et al, (2015) statement which states that the nucleotide sequence of the *acd*S gene is very close to the *dcyD* and *yedO* genes which code for the D-cysteine desulfhydrase enzyme.

Amino acids alignment of *acd*S gene of PCM8

The alignment of amino acid sequences of the *acd*S PCM8 gene with other bacteria aims to determine the conserved areas. Identical amino acid sequences are characterized by a black background as shown in Fig. 3. The catalytic residue of the *acd*S gene is Lys51 which plays a role in PLP binding, marked with a red square, the motive for the *acd*S gene sequence is marked with a yellow box. Other important residues, namely Ser78, Tyr 294, and Glu 295 which act to bind the ACC substrate are marked with a blue box.

Table 2: ANI result of PCM8 bacterial isolate

Strain	ANI	
	R. terrigena NBRC 14941	*R. terrigena* NCTC 13038
PCM8	98,88%	98,83%

Table 3: Annotation Results of PCM8 bacterial isolate

Id	KEGG Orthology	Description
LOCUS_19090	K01505	1-aminocyclopropane-1-carboxylate deaminase (E3.5.99.7)
LOCUS_19090	K05396	D-cysteine desulfhydrase (dcyD)

The alignment showed a low homology of 37,27% and there was a difference in the residue at the Glu295 position to Thr295. According to Betts and Russell (2003), glutamate residue can be substituted with threonine because it has the same polar properties and also has the same function as the binding site. The difference of important amino acid residues in PCM8 can affect the ability of the enzyme to bind to the substrate and also the type of substrate that is bound to the active site. The difference in amino acid residues led to differences in the ACCD activity of the two isolates. In this study, the differences in the position of Glu295 to Thr295 can increase ACCD activity.

Phylogenetic analysis

Phylogenetic analysis based on *acd*S gene and protein sequence from different microbial species has been conducted to study the evolution of ACCD gene (Fig. 4). According to Fig. 4, gene putative *acd*S of isolate PCM8 has closest similarity with *E. cloacae*, and followed by *Pseudomonas* sp. Ps 2-3, *Pseudomonas* sp. UW4 and *Ralstonia* sp. EcB11.

The amino acid sequence of the ACCD protein in PCM8 isolates was different from the other *acd*S sequences, this was because the *acd*S gene had high sequence diversity. Nascimento et al. (2014) inferred that the continuous vertical transmission of *acd*S genes might be responsible for the presence of *acd*S gene in bacteria that are not associated with ACC producing organism.

Physicochemical analysis of PCM8 *acd*S gene

The physicochemical analysis aimed to determine the character of the *acd*S gene for PCM8 strains, including the number of amino acid sequences, molecular weight, and pI value. This analysis was performed using the ProtParam prediction server (https://web.expasy.org/protparam/).

It was known that the *acd*S PCM8 gene had 325 amino acid sequences with a molecular weight of about ~ 34,95 kDa. This result is close to previous research which states that the ACCD enzyme measures around 35-42 kDa (Glick, 2005). This proves that the number of amino acids that make up the PCM8 *acd*S gene and their molecular weight values is in the range of the number of amino acids and the molecular weight of the *acd*S gene in ACCD-producing bacteria.

The properties of a protein can also be known from its isoelectric point. The isoelectric point is the pH where the number of positive and negative charges on the protein is balanced (Buxbaum, 2015). The isoelectric point is an important parameter in biochemical analysis and proteomic techniques (Kozlowski, 2016). The results of the analysis show that the *acd*S gene protein has an isoelectric point value of 5,06.4. These results are consistent with previous

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    ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----|
         5         15         25         35         45         55         65         75         85
1   MNLNRFERYP LTFGPSPITP LKRLSEHLGG KVELYAKRED CNSGLAFGGN KTRKLEYLIP EAIEQGCDTL VSIGGIQSNQ TRQVAAVAAH
2   --MNRFERYP LTFGPSPITP LKRLSERLGG KVELYAKRED CNSGLAFGGN KTRKLEYLIP EAIEQGCDTL VSIGGIQSNQ TRQVAAVAAH
3   MNLNRFERYP LTFGPSPITP LKRLSEHLGG KVELYAKRED CNSGLAFGGN KTRKLEYLIP EAIEQGCDTL VSIGGIQSNQ TRQVAAVAAH
4   MNLNRFERYP LTFGPSPITP LKRLSEHLGG KVELYAKRED CNSGLAFGGN KTRKLEYLIP EAIEQGCDTL VSIGGIQSNQ TRQVAAVAAH
5   MNLTRFPRLE LIGAPTPLEY LPRLSDHLG- -REIFIKRDD VTP-LAMGGN KLRKLEFLAA DALREGADTL ITAGAIQSNH VRQTAAVAAK
    ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----|
        95        105        115        125        135        145        155        165        175
1   LGMKCVLVQE NWVNYSDAVY DRVGNIEMSR IMGADVRLDA AGFDIGIRPS WEKAMSDVVE RGGKPFPIPA GCSEHPYGGL GFVGFAKELR
2   LGMKCVLVQE NWVNYSDAVY DRVGNIEMSR IMGADVRLDA AGFDIGIRPS WEKAMSDVVE RGGKPFPIPA GCSEHPYGGL GFVGFAEEVR
3   LGMKCVLVQE NWVNYSDAVY DRVGNIEMSR IMGADVRLDA AGFDIGIRPS WEKAMSDVVE RGGKPFPIPA GCSEHPYGGL GFVGFAEEVR
4   LGMKCVLVQE NWVNYSDAVY DRVGNIEMSR IMGADVRLDA AGFDIGIRPS WEKAMSDVVE RGGKPFPIPA GCSEHPYGGL GFVGFAEEVR
5   LSLHCVALLE NPIGTRAENY LTNGNRLLLD LFNTQVEMCD ALTDP--NAQ LEELATRIEA QGYRPYVIPV GGSS-ALGAL GYVESALEIA
    ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|----|
       185        195        205        215        225        235        245        255        265
1   QQEKDLGFKF DYIVVCSVTG STQAGMVVGF AADGRSKNVI GVDASAKPEQ TKAQILRIAR HTAELVELGR EITEEDVVLD TRFAYPEYGL
2   QQEKELGFKF DYIVVCSVTG STQAGMVVGF AADGRSKNVI GVDASAKPEQ TKAQILRIAR HTAELVELGR EITEEDVVLD TRFAYPEYGL
3   QQEKELGFKF DYIVVCSVTG STQAGMVVGF AADGRSKNVI GVDASAKPEQ TKAQILRIAR HTAELVELGR EITEEDVVLD TRFAYPEYGL
4   QQEQALGFKF DYIVVCSVTG STQAGMVVGF AADGRSKNVI GIDASAKPEK TRAQILRIAR HTAELVELGR EITEEDVVLD TRFAYPEYGL
5   QQCEG-AVEL SSVVVASGSA GTHAGLAVGL EQLMPGAELI GVTVSRKVAD QLPKVVALQQ AVANSLELQ- --AKAGITLW DDYFAPGYGT
    ----|----| ----|----| ----|----| ----|----| ----|----| ----|----| ----|---
       275        285        295        305        315        325        335
1   PNEGTLEAIR LCGSLEGVLT DPVYEGKSMH GMIEMVRRGE FPDGSKVLYA HLGGAPALNA YSFLFRNG
2   PNEGTLEAIR LCGSLEGVLT DPVYEGKSMH GMIEMVRRGE FPDGSKVLYA HLGGAPALNA YSFLFRNG
3   PNEGTLEAIR LCGSLEGVLT DPVYEGKSMH GMIEMVRRGE FPDGSKVLYA HLGGAPALNA YSFLFRNG
4   PNDGTLEAIR LCGRLEGVLT DPVYEGKSMH GMIEMVRRGE FPEGSKVLYA HLGGVPALNA YSFLFRNG
5   PNDEGMEAVK LLAQLEGILL DPVYTGKAMA GLIDGIQQKR FKDEGPILFI HTGGAPALFA YHPHI---
```

Fig 3. The result of the alignment of the amino acid sequence of the *acd*S gene with several bacteria. (note: 1. *Klebsiella oxytoca,* 2. *Serratia rubiadeae*, 3. *Pseudomonas putida* UW 4*,* 4. *Pseudomonas fluorescens* KACC10070, 5. PCM8.

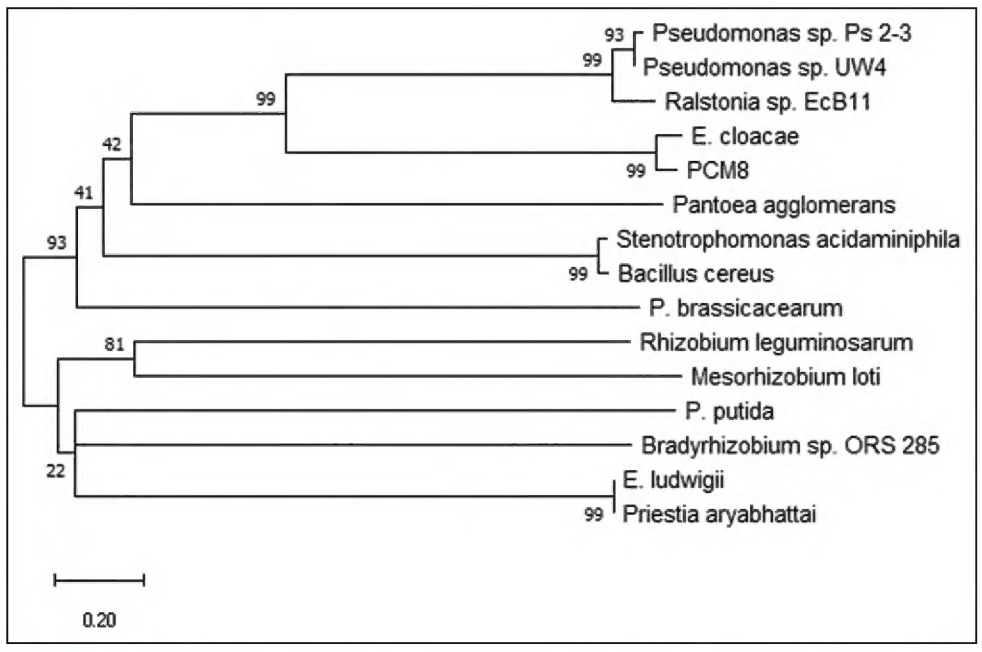

Fig 4. The phylogenetic tree constructed from *acd*S amino acid sequence of different bacterial strains.

research which shows that the isoelectric point value of the *acd*S gene protein ranges from 4,63 to 6,51 (Pramanik et al., 2017).

Tertiary structure analysis of PCM8

The tertiary structure analysis and protein modeling of the *acd*S gene for PCM8 strains were carried out to determine the shape, location of the active site of the protein, and its similarity to the protein structure of the comparator *acd*S gene. The tertiary structure is formed by the interaction between the branch chains of the secondary structure and the amino acid residues. The tertiary structure is a combination of various interactions, including hydrophobic interactions, disulfide bonds, hydrogen bonds, and ionic bonds. In this analysis, the amino acid sequence *Pseudomonas putida* UW4 was used as a comparison with a sequence identity value of 61,86% as shown in Fig. 5. Sequence identity values of more than 50% can be used for modeling because they have small errors in predicting three-dimensional structures, namely in the side chain and rotameric area (Baker and Sali, 2001).

Fig. 5.(A) showed that the protein structure of the *acd*S PCM8 gene was almost parallel to the protein structure of the *acd*S *Pseudomonas putida* UW4 gene with an RMSD value of 1.422. The RMSD value shows the similarity of a protein, if the RMSD value between the two protein structures is getting smaller, the structure of the two proteins will be more similar.

The Lysine 51 (Lys51), Serine 78 (Ser78), and Tyrosine294 (Tyr294) residues of PCM8 have similar orientation to the Lysine 51 (Lys51) Serin 78 (Ser78), and Tyrosin287 (Tyr287) residues of *Pseudomonas putida* UW4. These results sugessted that the *acd*S gene for PCM8 strain had the same catalytic residues, except for the Gultamate295 (Glu295) found to be mutated into Threonine288 (Thr288) in PCM8. Todorovic and Glick (2008) found that the amino acid mutation Glu295 in *Pseudomonas* sp. UW4 causes decreased ACCD enzyme activity but shows increased D-cysteine desulfhydrase activity. This causes the bacteria to be able to utilize substrates other than ACC. By having a broad ability to break down ACC-like substrates and some D-amino acids, the *acd*S gene can be maintained by microorganisms even when growing in an ACC-free environment. Bacteria that have the ACCD enzyme are

included in the PLP-dependent deaminase group associated with the tryptophan beta-synthase subunit and have the same origin (Nascimento, 2014).

Analysis of Ramachandran plots through the SWISS-MODEL Procheck was required to determine the quality of the protein structure (Fig. 6).

The results of the Ramachandran plot showed that the percentage of amino acid residues in the favored region is 91%. According to Lovell et al (2003), a good protein structure if it has a percentage of amino acids in the favored region of more than 90%, and the residue in the outlier area is less than 15% so it can be concluded that the PCM8 *acd*S protein has good and stable structural quality.

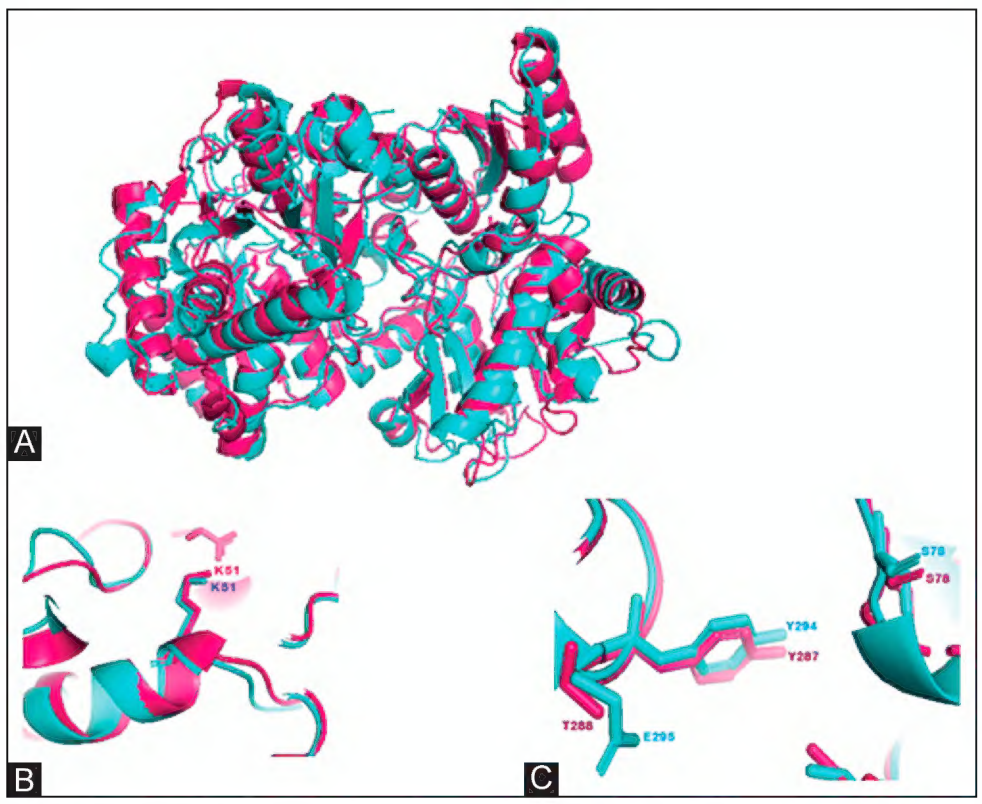

Fig 5. Superposition between the PCM8 and *Pseudomonas putida* UW4 *acd*S gene product (protein). (A) Global structure of the tertiary structure of the *Pseudomonas putida* UW4 (blue strands) and the PCM8 (purple strands) *acd*S gene product (protein). Modeling was carried out using SWISSMODEL and visualized by PyMol software. The RMSD value was 1.422. (B) The orientation of residue that binds PLP (Lys51) and (C) The orientation of ligand binding residues of *acd*S gene product of *Pseudomonas putida* UW4 (Ser78-Tyr 294-Glu295) and PCM8 (Ser78-Tyr287-Thr288).

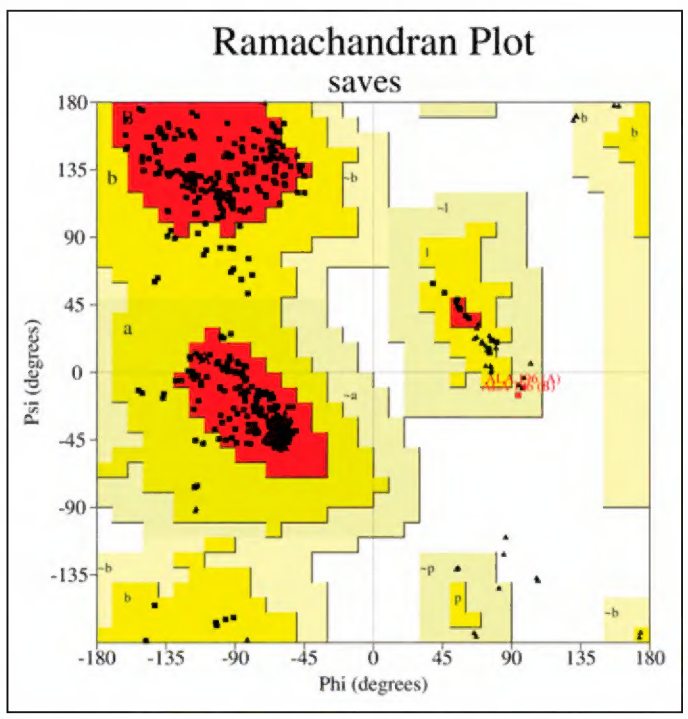

Fig 6. Ramachandran Plot of the *acd*S gene product of PCM8 strains. The percentage of amino acid residues in the most favored region was 91%, residues in the additional allowed region were 8,7%, the residues in the generally allowed region were 0,4% and amino acid residues in the disallowed region were 0%.

Nitrogenase activity

The nitrogenase activity of the PCM8 strains and *Pseudomonas putida* UW4 was measured by the ARA (*Acetylene Reduction Assay*) method by measuring the amount of ethylene formed per gram of bacteria per hour. The results of nitrogenase activity in the selected strains presented in Table 4.

Based on the previous study, the PCM8 strain had the higher nitrogenase activity compared to other strains. Comparison of nitrogenase activity of PCM8 strains with UW4 control strain was analyzed using *Paired T-Test* to predict the potential of selected strains in fixing nitrogen. Based on statistical analysis, strain of PCM8 had significantly higher nitrogenase activity than UW4. On the other hand, it shows that these strains have potential as nitrogen-fixing organisms.

The *nif*H gene of PCM 8 isolate

The amplification of the *nif*H target gene using genomic DNA of PCM8 isolate and the primary degenerate. The degenerate primer amplifies the *nif*H target gene by about 300 bp. Isolate PCM8 was suspected of having a *nif*H target gene based on PCR and electrophoresis results. Through the blastn and blastx alignment program from the sequencing results, isolate *R. terrigena* PCM 8 was identified as having a *nif*H gene (dinitrogenase reductase) with 87,88% similarity level, that confirming the nitrogenase activity by the PCM8 isolate.

Double ACC deaminase and nitrogenase activity

Based on the results of ACCD and nitrogenase activity analysis above, PCM8 strain has a double activity that has high activity both on ACCD and nitrogenase activity compared to *P. putida* UW4 strains as a role model of ACCD bacteria (Glick et al., 1995). The superiority of bacteria with double capability of ACCD and nitrogenase has been revealed by Hassan et al. (2015). The WAN1 strain having ACCD and nitrogenase activity can increase root elongation and maximum stem in wheat crops compared with strains that only have ACCD activity or nitrogen fixation. Meanwhile in another study, Hassan et al. (2016) reported that SAN1 strains having ACCD activity and nitrogen fixation were able to increase high plant germination compared to other strains that have only ACCD activity or nitrogen fixation under the heavy metal Cd (Cadmium) stress. The strain PCM8 can be potential organism to increase germination and roots elongation of plant stems under stress conditions.

Table 4: Nitrogenase Activity of ACCD producing bacteria and Positive control *P. putida* UW4. The assay using Gas Chromatography

No.	Bacterial Strain	Nitrogenase Activity (nmol C_2H_4/gr cell/hour)
1	*Raoultella* sp. PCM 8	61,05±3,04[b]
2	*P. putida* UW4	8,07±2,69[a]

Values are the average from three replicates. The standard deviation was also shown. Values with the different superscripts within same column indicate significant difference with P ≤0.05.

PCM8 strains have high nitrogenase activity but ACCD activity is lower compared to *P. putida* UW4 strain. This proves that: 1) not all bacteria that have ACCD activity have nitrogenase activity and conversely. 2) ACCD activity is not affected by nitrogenase activity although ACCD regulation is affected by the *nifA*2 gene and conversely. This is in accordance with the reported Han et al. (2015) that *acdS* expression, the encoding gene of ACCD, does not increase despite the expression of *rpo*N and *nif*A, the regulatory gene in nitrogenase enzyme, is enhanced. In another study conducted by Nascimento et al. (2016) reported that ACCD activity had no effect on nitrogenase activity, but ACCD was involved in the early stages of nodular formation but not in nodular function (nitrogen fixation).

CONCLUSIONS

In conclusion, *Raoultella terrigena* PCM8 is a bacterial strain that has the higher ACCD and nitrogenase double activity compared to the *Pseudomonas Putida* UW4. The ACCD enzyme has a molecular weight of ~ 34,95 kDa with pI value of 5,06, and catalytic residues consist of Lys51, Ser78, Tyr287, and Thr288.

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